Supporting Information for:
A Perturbed pK_a at the Binding Site of the Nicotinic Acetylcholine Receptor: Implications for Nicotine Binding

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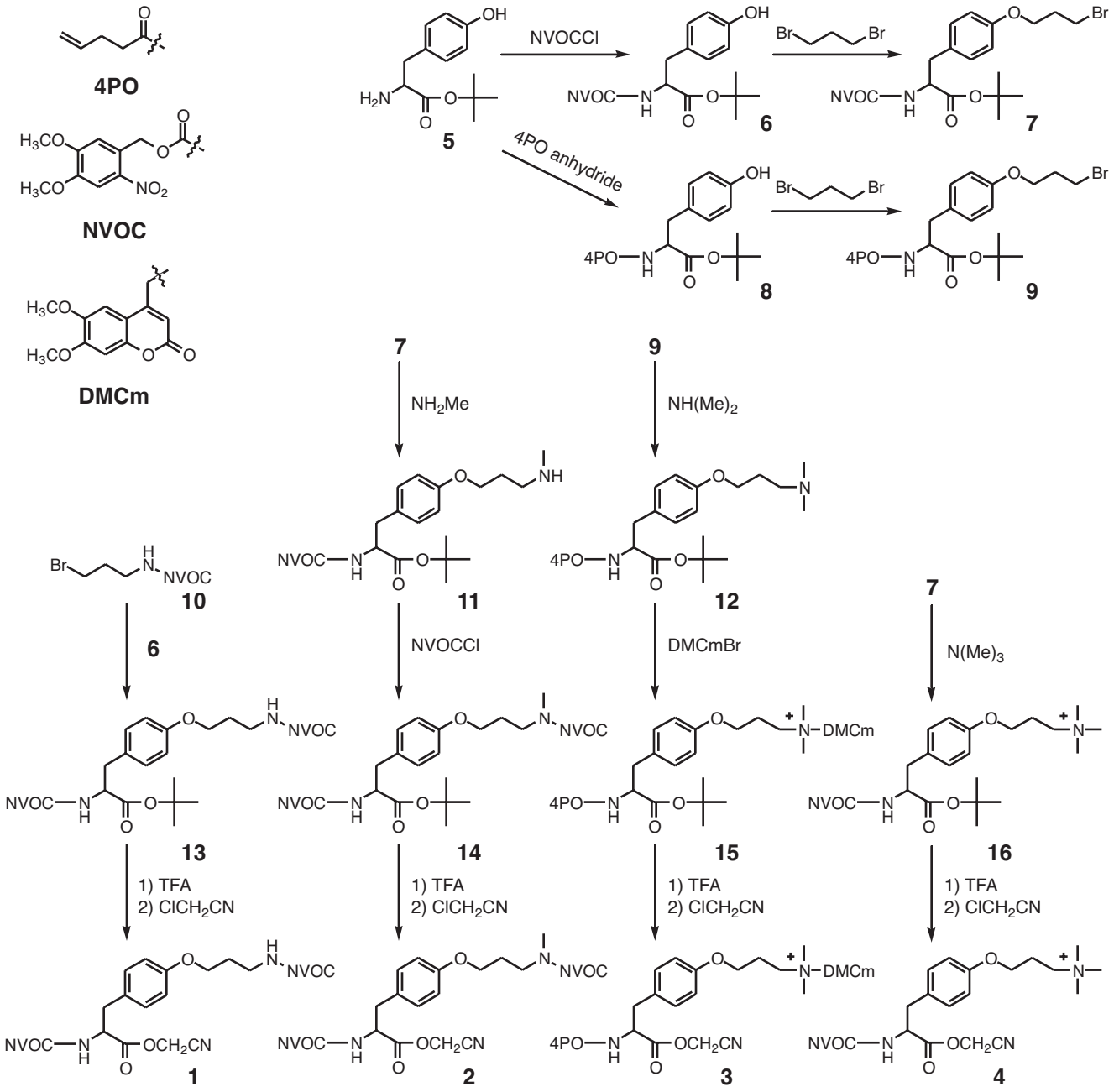

Scheme A. Syntheses of TyrO3P, TyrO3S, TyrO3T, and TyrO3Q precursors; compounds **1**, **2**, **3**, and **4**, respectively. Cyanomethyl esters **1**, **2** , **3**, and **4** are prepared for coupling to dCA and subsequent enzymatic ligation to tRNA. The synthesis of **4** has been published previously.[1]

Experimental Procedures

General. Reagents were purchased from Aldrich, Sigma, or other commercial sources. TMB-8 was purchased from RBI (Natick, MA). ACh chloride and QX-314 were purchased from Sigma. Anhydrous THF and methylene chloride were obtained from J. T. Baker solvent kegs; anhydrous DMF (Puris) was obtained from Fluka. Flash chromatography was on 230-400 mesh silica gel with the solvent indicated. All NMR shifts are reported as δ ppm downfield from TMS. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz in $CDCl_3$ or CD_3CN using a Varian QE-300 spectrometer. Electrospray (ESI) ionization and matrix-assisted laser-desorbed ionization (MALDI) quadrupole mass spectrometry was performed at the Caltech Protein/Peptide Micro Analytical Laboratory or at the Caltech Division of Chemistry and Chemical Engineering Mass Spectrometry Facility. Nitroveratryloxycarbonyl chloride (NVOCCl), NVOC-Tyrosine *t*-butyl ester (NVOC-Tyr-O*t*Bu, **6**), and NVOC-Tyrosine-*O*-propylbromide *t*-butyl ester (NVOC-TyrO3Br-O*t*Bu, **7**) were prepared as previously described.[1]

***N*-NVOC-Bromopropylamine (10).** 0.15 g 3-bromopropylamine were dissolved in 15 mL *p*-dioxane and 15 mL water. 0.11 g Na_2CO_3 were dissolved in 15 mL water. 3-bromopropylamine solution was combined with the Na_2CO_3 solution. 0.19 g NVOC-Cl were completely dissolved in 25 mL dioxane, and were added to the other mixture and stirred for 1 h. The reaction mixture was extracted with 3 X 25 mL CH_2Cl_2, the organic layers were combined, dried with sodium sulfate, and run on a column in methylene chloride to give 0.25 g (37%) of product. ^{1}H NMR ($CDCl_3$): δ 7.66 (s, 1H), 6.97 (s, 1H), 5.46 (s, 2H), 5.16 (br s, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.43 (t, J=6.3 Hz, 2H), 3.36 (td, J=6.3 Hz, 6.3 Hz, 2H), 2.07 (m, 2H); ^{13}C NMR ($CDCl_3$): 155.7, 153.2, 147.8, 127.7, 110.1, 107.9, 63.5, 56.3, 56.3, 39.4, 32.3, 30.6.

NVOC-TyrO3P(NVOC)-O*t*Bu (13). To a mixture of 0.12 g NVOC-Tyr-O*t*Bu and 0.166 g Cs_2CO_3 (two equivalents) dissolved in 10 mL DMF were added 0.096 g *N*-NVOC-bromopropylamine in 13 mL of anhydrous DMF, under Ar. After the reaction was stirred for 1.5 hours, the reaction mixture was extracted using 3 X 25 mL methylene chloride, the organic layers were combined, dried with sodium sulfate, and run on a flash column in 1:1 petroleum ether / ethyl acetate to give 75 mg (38%) of product. ^{1}H NMR ($CDCl_3$): δ 7.70 (s, 1H), 7.49 (s, 1H), 7.08 (d, J=4.2 Hz, 2H), 7.00 (s, 1H), 6.97 (s, 1H), 6.81 (d, J=4.2 Hz, 2H), 5.52 and 5.47 (AB, J=11.4 Hz, 11.4 Hz, 2H), 5.50 (s, 2H), 5.41 (d, 1H), 5.26 (br s, 1H), 4.52 (m, 1H), 4.02 (t, J=5.7 Hz, 2H), 4.98 (s, 3H), 4.98 (s, 3H), 4.98 (s, 3H), 4.98 (s, 3H), 3.44 (dt, J=6.3 Hz, 6.3 Hz, 2H), 3.05 (m, 2H), 2.02 (m, 2H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$): 170.4, 157.4, 155.7, 155.0, 153.4, 153.2, 147.7, 147.5, 141.0, 139.2, 130.2, 128.1, 128.0, 114.2, 114.0, 109.9, 109.5, 107.9, 107.9, 82.3, 65.6, 63.6, 63.4, 56.4, 56.3, 56.1, 55.2, 38.7, 37.2, 29.3, 27.9.

NVOC-TyrO3P(NVOC)-OCH$_2$CN (1). 0.075 g NVOC-Tyr-O3P(NVOC)-O*t*Bu were dissolved in 5 mL methylene chloride. 3 mL TFA were added to the mixture, using a glass pipette. The reaction was stirred for 1 hour. Volatiles were removed on the vacuum pump with a dry ice/acetone trap. 10 mL anhydrous DMF, 5 mL chloroacteonitrile, and 1 mL diisopropylethylamine (DIPEA) were added to the flask, under argon. After the reaction was stirred for 3 hours, the volatiles in the mixture were removed on the vacuum pump with a dry ice/acetone trap. The reaction mixture was run on a silica column in 1:1 petroleum ether / ethyl acetate to give 53 mg (72%) product. ^{1}H NMR ($CDCl_3$): 7.70 (s, 1H), 7.70 (s, 1H), 7.07 (d, J=4.2 Hz, 2H), 7.01 (s, 1H), 6.94 (s, 1H), 6.85 (d, J=4.2 Hz, 2H), 5.53 and 5.50 (AB, J=14.7 Hz, 14.8 Hz, 2H), 5.50 and 5.47 (AB, J=15.1 Hz, 14.7 Hz, 2H), 5.41 (s, 1H), 5.28 (d, J=6.3 Hz, 1H), 4.78 and 4.70 (AB, J=21.3 Hz, 21.3 Hz, 2H), 4.69 (m, 1H), 4.03 (t, J=5.7 Hz, 2H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.44 (dt, J=6.3 Hz, 6.3 Hz, 2H), 3.10 (m, 2H, J=6.3 Hz, 6.3 Hz), 2.02 (m, J=6.3 Hz, 2H); ^{13}C NMR ($CDCl_3$): 170.2, 157.8, 155.7, 155.0, 153.3, 153.2, 147.8, 147.6, 141.0, 139.4, 130.0 127.9, 127.4, 126.7, 114.6, 113.5, 110.0, 109.8, 107.9, 65.6, 64.0, 63.4, 56.4, 56.2, 56.3, 56.1, 54.8, 48.9, 38.6, 36.9, 29.7, 29.2.

NVOC-TyrO3S-O*t*Bu (11). 268 mg NVOC-TyrO3Br-O*t*Bu were dissolved in 20 mL anhydrous THF under Ar in a 3-neck round-bottom flask with a CO_2(s) / acetone condenser. This was cooled to - 20 °C in a 30% KCl / ice bath and then NH_2Me gas was bubbled through the solution until the drip rate from the condenser tip was about 2 s^{-1}. The setup was allowed to warm to RT and then the reaction mixture was rotoevaporated to ensure removal of the dissolved NH_2Me. Chromatographic purification was achieved by elution of the starting material in CH_2Cl_2 followed by collection of the product in CH_2Cl_2 with 5% triethylamine. 164 mg of flaky yellow solid (67%) were obtained in this manner. ^{1}H NMR ($CDCl_3$) δ 7.67 (s, 1H), 7.09 (d, J = 8.5 Hz, 2H), 6.96 (s, 1H), 6.81 (d, J = 8.5 Hz, 2H), 5.81 (d, J = 8.1 Hz, 1H), 5.52 and 5.44 (AB, J = 15.2 Hz, 2H), 4.49 (m, 1H), 3.99 (t, J = 6.3 Hz, 2H), 3.95 (s, 3H), 3.93 (s, 3H), 3.03 (m, 2H), 2.75 (t, J = 6.9 Hz, 2H), 2.44 (s, 3H), 1.95 (m, 2H), 1.43 (s, 9H)

NVOC-TyrO3S(NVOC)-O*t*Bu (14). 65 mg Na_2CO_3 (2 equiv.) were dissolved in 15 mL water and added to a solution of 164 mg NVOC-TyrO3S-O*t*Bu in 15 mL *p*-dioxane. 168 mg NVOC-Cl (2 equiv.) in 15 mL *p*-dioxane were added to this and the reaction was stirred overnight. The product was purified by flash chromatography in 1:1 EtOAC / petroleum ether, giving 104 mg (44%) of a dark yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.72 (s, 1H), 7.68 (s, 1H), 7.07 (d, J = 8.4 Hz, 2H), 7.03 (s, 1H), 7.01 (s, 1H), 6.79 (d, J = 8.4 Hz, 2H), 5.56 and 5.51 (AB, J = 15.0 Hz, 2H), 5.47 (br s, 2H), 5.35 (d, J = 7.5 Hz, 1H), 4.51 (m, 1H), 3.96 (br t, J = 6.3 Hz, 2H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.95 (s, 3H), 3.52 (t, J = 12.5 Hz, 2H), 3.05 (m, 2H), 3.00 (d, J = 17.5 Hz, 2H), 2.05 (m, 2H), 1.44 (s, 9H)

NVOC-TyrO3S(NVOC)-OCH$_2$CN (2). After 64 mg NVOC-TyrO3S(NVOC)-O*t*Bu were stirred with 1.00 mL trifluoroacetic acid for 1 hr. in 10 mL CH_2Cl_2, the reaction mixture was pumped on for 2 h. with a dry ice / acetone trap. The crude amino acid was coevaporated with toluene and redissolved in 10 mL DMF under Ar for esterification. 0.50 mL chloroacetonitrile was added with 0.10 mL DIPEA and the mixture was stirred at RT. The next morning, the volatiles were removed under vacuum and the mixture was run on a column in 3:1 petroleum ether / EtOAc. 24 mg of an orange solid were obtained in a 40% yield. ^{1}H NMR ($CDCl_3$) δ 7.70 (s, 1H), 7.67 (s, 1H), 7.07 (d, J = 8.4 Hz, 2H), 7.03 (s, 1H), 6.99 (s, 1H), 6.81 (d, J = 8.4 Hz, 2H), 5.55 and 5.49 (AB, J = 15.1 Hz, 2H), 5.47 (br d, J = 15.1 Hz, 2H), 5.33 (d, J = 7.5 Hz, 1H), 4.98 (br s, 2H), 4.38 (m, 1H), 3.97 (br t, J = 6.3 Hz, 2H), 3.94 (s, 3H), 3.94 (s, 3H), 3.94 (s, 3H), 3.94 (s, 3H),

3.56 (t, J = 10.5 Hz, 2H), 3.04 (m, 2H), 2.98 (d, J = 16.0 Hz, 2H), 2.08 (m, 2H), 1.46 (s, 9H)

4PO-Tyr-O*t*Bu (8). 148 mg Na_2CO_3 (1.4 equiv.) were stirred with 237 mg Tyr-O*t*Bu in 25 mL H_2O and 20 mL *p*-dioxane. 256 µL 4-pentenoic anhydride (4P) anhydride, 2.1 equiv., Aldrich), dissolved in 5 mL *p*-dioxane, were added. The reaction was quenched after 1 hr. with 25 mL each CH_2Cl_2 and 1 M $NaHSO_4$. The aqueous layer was extracted with 3 X 25 mL CH_2Cl_2 and purified on a column in 3:1 petroleum ether / EtOAc. 249 mg of an oily yellow solid obtained in a 73% yield. ^{1}H NMR ($CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 2H), 6.68 (d, J = 8.5 Hz, 2H), 6.21 (d, J = 6.6 Hz, 1H), 5.66 (m, 1H), 4.94 (d, J = 18.0 Hz, 1H), 4.89 (d, J = 11.2 Hz, 1H), 4.66 (m, 1H), 2.90 (m, 2H), 2.18 (m, 2H), 2.21 (m, 2H), 1.37 (s, 9H) ^{13}C NMR ($CDCl_3$): 171.6, 171.2, 155.6, 139.3, 130.5, 128.5, 115.5, 114.5, 82.6, 53.4, 37.5, 35.7, 32.6, 28.2.

4PO-TyrO3Br-O*t*Bu (9). 750 mg Cs_2CO_3 (2 equiv.) were combined with 2.00 mL 1,3-dibromopropane in 20 mL dry DMF in a flame-dried flask under Ar. 249 mg 4PO-Tyr-O*t*Bu were dissolved in 10 mL DMF under Ar, added to the Cs_2CO_3 slurry through a septum, and stirred overnight. The reaction mixture was stirred 10 min. with 30 mL water and extracted with 3 X 25 mL CH_2Cl_2. Flash chromatography of the combined organics with 3:1 petroleum ether / EtOAc gave 157 mg of sticky yellow solid, a 47% yield. ^{1}H NMR ($CDCl_3$) δ 7.00 (d, J = 8.9 Hz, 2H), 6.75 (d, J = 8.7 Hz, 2H), 6.24 (d, J = 6.8 Hz, 1H), 5.68 (m, 1H), 4.96 (d, J = 17.9 Hz, 1H), 4.91 (d, J = 11.3 Hz, 1H), 4.67 (m, 1H), 3.99 (t, J = 5.8 Hz, 2H), 3.51 (t, J = 6.6 Hz, 2H), 2.94 (m, 2H), 2.27 (m, 2H), 2.23 (m, 2H), 2.20 (m, 2H), 1.36 (s, 9H) ^{13}C NMR ($CDCl_3$): 171.8, 171.1, 157.8, 137.1, 130.7, 128.7, 115.7, 114.5, 82.4, 65.5, 53.8, 37.5, 35.9, 32.7, 30.4, 29.7, 28.3.

4PO-TyrO3T-O*t*Bu (12). 236 mg 4PO-TyrO3Br-O*t*Bu were dissolved in 25 mL dry THF in a 3-neck round-bottom flask with a dry ice / acetone condenser under Ar. The setup was cooled in ice and $NH(Me)_2$ gas was bubbled through the yellow solution until it was dripping vigorously from the condenser tip. The cooling apparatus was maintained for 2 hrs. and then the reaction mixture was allowed to warm to RT. After rotoevaporation to remove the volatile components, the mixture was chromatographed. Starting material was eluted with EtOAc and then the product was collected with 5% triethylamine in CH_2Cl_2, giving 210 mg (96%) of a sticky yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.01 (d, J = 8.0 Hz, 2H), 6.79 (d, J = 8.0 Hz, 2H), 5.95 (d, J = 7.2 Hz, 1H), 5.76 (m, 1H), 5.02 (d, J = 17.7 Hz, 1H), 4.97 (d, J = 12.3 Hz, 1H), 4.71 (m, 1H), 3.96 (t, J = 6.3 Hz, 2H), 3.00 (m, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 9.0 Hz, 2H), 2.20 (s, 6H), 1.93 (m, 2H), 1.35 (s, 9H), 1.27 (m, 2H) ^{13}C NMR ($CDCl_3$): 171.3, 170.6, 157.7, 136.7, 130.2, 127.8, 115.3, 114,1, 82.0, 66.0, 56.3, 53.4, 45.5, 37.0, 35.6, 29.3, 27.9, 27.5.

4PO-TyrO3T(DMCm)-O*t*Bu (15). 762 mg **12** and 675 mg dimethoxycoumarinmethylbromide (DMCmBr) were stirred overnight in 200 mL CH_3CN at 60 °C under Ar. After rotoevaporation, the reaction mixture was purified by flash chromatography on silica gel. First, starting material was eluted using 1:1 petroleum ether / EtOAc with 5% MeOH, then product was eluted with 7:1:1:1 EtOAc / MeOH / AcOH / H_2O. 857 mg of an intensely yellow acetate salt were obtained in a 67% yield. ^{1}H NMR ($CDCl_3$) δ 7.58 (s, 1H), 7.05 (d, J = 9.4 Hz, 2H), 6.86 (s, 1H), 6.78 (d, J = 9.6 Hz, 2H), 6.63 (s, 1H), 6.11 (d, J = 7.4 Hz, 1H), 5.79 (m, 1H), 5.20 (br s, 2H), 5.04 (d, J = 18.0 Hz, 1H), 4.99 (d, J = 10.0 Hz, 1H), 4.72 (m, 2H), 4.05 (t, J = 8.2 Hz, 2H), 3.96 (s, 3H), 3.94 (s, 3H), 3.88 (br t, 2H), 3.30 (s, 6H), 3.01 (m, 2H), 2.35 (t, J = 7.8 Hz, 2H), 2.31 (m, 2H), 2.28 (m, 2H), 2.05 (s, 3H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$): 177.1, 171.6, 170.6, 159.6, 156.8, 153.6, 149.9, 147.0, 142.0, 136.6, 130.4, 128.9, 119.8, 115.4, 114.0, 111.1, 105.9, 100.1, 82.3, 64.0, 63.1, 62.2, 56.9, 56.4, 53.5, 51.0, 37.1, 35.5, 29.3, 28.0, 23.2, 22.1.

4PO-TyrO3T(DMCm)-OCH$_2$CN (3). 857 mg 4PO-TyrO3T(DMCm)-O*t*Bu were dissolved in 25 mL methylene chloride with. 5 mL TFA. When reaction was complete, volatiles were removed on the vacuum pump with a dry ice / acetone trap. 15 mL anhydrous $ClCH_2CN$ and 1 mL triethylamine were added to the flask, under argon. After stirring overnight, the volatiles in the mixture were removed on the vacuum pump with a dry ice/acetone trap. The reaction mixture was run on a column in 10:1:1:1 EtOAc / MeOH / AcOH / H_2O. acetate. Collected fractions were rotovapped to dryness, redissolved in minimal CH_2Cl_2, and extracted against H_2O to remove triethylamine salt. 651 mg of an acetate salt, a 78% yield. ^{1}H NMR ($CDCl_3$) δ 7.73 (s, 1H), 6.98 (d, J = 8.5 Hz, 2H), 6.79 (s, 1H), 6.70 (d, J = 8.5 Hz, 2H), 6.65 (s, 1H), 6.11 (d, J = 7.3 Hz, 1H), 5.77 (m, 1H), 5.44 (br s, 2H), 5.03 (d, J = 15.0 Hz, 1H), 4.97 (d, J = 10.5 Hz, 1H), 4.81 (m, 2H), 4.04 (t, J = 6.8 Hz, 2H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (t, J = 6.7 Hz, 2H), 3.42 (s, 6H), 3.07 (m, 2H), 2.42 (m, 2H), 2.36 (m, 2H), 2.31 (m, 2H), 2.02 (s, 3H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$): 177.1, 172.2, 170.2, 159.5, 157.9, 163.6, 149.8, 147.1, 141.8, 136.6, 130.1, 128.0, 120.2, 115.4, 114.5, 114.0, 111.0, 106.2, 100.0, 64.1, 63.5, 62.1, 57.6, 56.4, 53.2, 50.5, 48.8, 36.6, 25.0, 29.2.

General procedure for coupling of cyanomethyl esters to dCA. The dinucleotide was prepared as previously reported[2] with a few modifications: 1) the dinucleotide coupling, oxidation, and deprotection with *p*-toluenesulfonic acid were done in one pot; 2) the desalting of dCA was accomplished by redissolving it in Millipore water, freezing, and lyophilization to obtain a fluffy material; 3) the tetrabutylammonium salt of the dinucleotide was formed by mixing the proper amount 1 M N(*n*-Bu)$_4$OH in MeOH with a solution of dCA in water. Freezing and lyophilization provided a white fluffy solid which was then stored at -80 °C.

NVOC-TyrO3P(NVOC)-OdCA. The synthesis of NVOC-TyrO3P(NVOC)-OdCA is described as a general procedure. 23 mg of dCA (tetrabutylammonium salt) were dissolved in 0.5 mL anhydrous DMF, and were stirred with 53 mg NVOC-TyrO3P(NVOC)-OCH$_2$CN (four equivalents) dissolved in 0.5 mL anhydrous DMF in a 10 mL pear-shaped flask, under argon. After 4 hours, a small amount of tetrabutylammonium acetate was added using a metal spatula. The reaction was monitored by analytical HPLC, using a Waters NOVA-Pak C_{18} (150 x 3.9 mm) reverse-phase column with a gradient from 25 mM NH_4OAc (pH 4.5) to CH_3CN. When the reaction was judged complete after 24 hours, the mixture was purified by semi-preparative HPLC with a Waters NOVA-Pak C_{18} (300 x 7.8 mm) using a similar gradient. The appropriate fractions were combined, frozen, and lyophilized overnight. To remove ammonium ions, which inhibit T4 RNA ligase in the ligation of the product to tRNA, the product was redissolved in 10 mM acetic acid, frozen, and lyophilized again. This yielded 5.6 mg (6%; yields can be as low as 1%) of the desired product as a pale yellow solid. Small amounts of material were quantified by their UV-Vis spectra in solution of 10 mM acetic acid, assuming ε_{350} = 6336 M^{-1} per nitroveratryl group. ESI$^+$-MS: calculated for 755.7; found [M+Na]$^+$: 778.4.

NVOC-Tyr-O3S(NVOC)-dCA. Coupling procedure differed in that N(*n*-Bu)$_4$OAc salt was added to a cloudy mixture of dCA in 500 µL DMF until dissolution was complete and then this was combined with a 500 µL solution of the cyanomethyl ester in DMF. Reaction was complete after 2 h. 2 mg obtained in a 5% yield. from 24 mg of cyanomethyl ester and 32 mg dCA. ESI$^+$-MS: calculated for $C_{52}H_{63}N_{12}O_{27}P_2^+$: 1349.3 ; found $[M+H]^+$: 1349.3

4PO-TyrO3T(DMCm)-dCA. Prepared by the general coupling procedure. After 36 h., reaction appeared to have reached steady-state at ~ 60% completion. 6 mg obtained in a 31% yield. from 16 mg of cyanomethyl ester and 20 mg dCA. Quantification was performed assuming ε_{350} = 13,000 M^{-1} for the coumarin group. ESI$^+$-MS: calculated for $C_{50}H_{63}N_{10}O_{20}P_2^+$: 1185.4 ; found $[M+]^+$: 1185.4

Note: 4PO-TyrO3T(DMCm)-dCA was originally synthesized with a nitroveratryl (NV) protecting group on its side-chain amine. However, even after 1 h of photolysis under the above conditions, no cleavage of the NV group was observed, as detected by tandem HPLC electrospray MS (positive ion, in 25 mM NH_4OAc). Photolysis of the model compound *N*-NV-3-dimethylaminopropanol also failed, even after 1 h of irradiation. Deprotection of the DMCm-protected 3° amine, on the other hand, was complete after 5 - 10 min.

Unnatural amino suppression in *Xenopus* oocytes. The site-directed mutagenesis of the nAChR TAG mutants, gene construction and synthesis of suppressor tRNA and ligation of aminoacyl-dCA to tRNA have been described previously[2]. Plasmid DNAs were linearized with NotI, and mRNA was transcribed using the Ambion (Austin, TX) T7 mMESSAGE mMACHINE Kit.

Oocytes were removed from *Xenopus laevis* as described[4] and maintained at 18 °C, in ND96 solution (96 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/5 mM HEPES/2.5 mM sodium pyruvate/0.5 mM theophyline/10 g/ml Gentamycin, pH 7.5, with NaOH). Before microinjection, the NVOC-aminoacyl-tRNA was deprotected by irradiating the sample for 5 or 10 min. with a 1000 W Hg/Xe arc lamp (Oriel) operating at 400 W equipped with WG-335 and UG-11 filters (Schott). 4PO-protected tRNA-aa was mixed 1:1 with a solution of saturated I_2 in water and allowed to sit for ten minutes at room temperature. Each oocyte was injected with a 1:1 mixture of deprotected aminoacyl-tRNA (25-50 ng) and mRNA (12.5–18 ng of total at a concentration ratio of 20:1:1:1 for α:β:γ:δ subunits) in a volume of 50 nL.

Electrophysiological recordings. Voltage-clamped electrophysiological recordings were carried out 24-72 hours after injection. Whole-cell currents from oocytes were measured using a Geneclamp 500 amplifier and pCLAMP software (Axon Instruments, Foster City, CA) in the two-electrode voltage-clamp configuration. Microelectrodes were filled with 3 M KCl and had resistances ranging from 1.0 to 2.5 MΩ. Oocytes were continuously perfused with a nominally Ca^{2+}-free bath solution consisting of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.5). Microscopic ACh-induced and TMB-8 or QX-314-blocked currents were recorded in response to bath application of ACh and TMB-8 at a holding potential of -80 mV. Low (5.5 - 6.5) and high (8.5 and 9.0) pH solutions were of the same composition as Ca^{2+}-free bath with MES (low) or CHES (high) substituted for HEPES buffer. To ensure that changes in buffer were not responsible for the observed changes in channel conductance, recordings were taken at pH 7.0 and 8.0 in HEPES alongside recordings in MES and CHES.

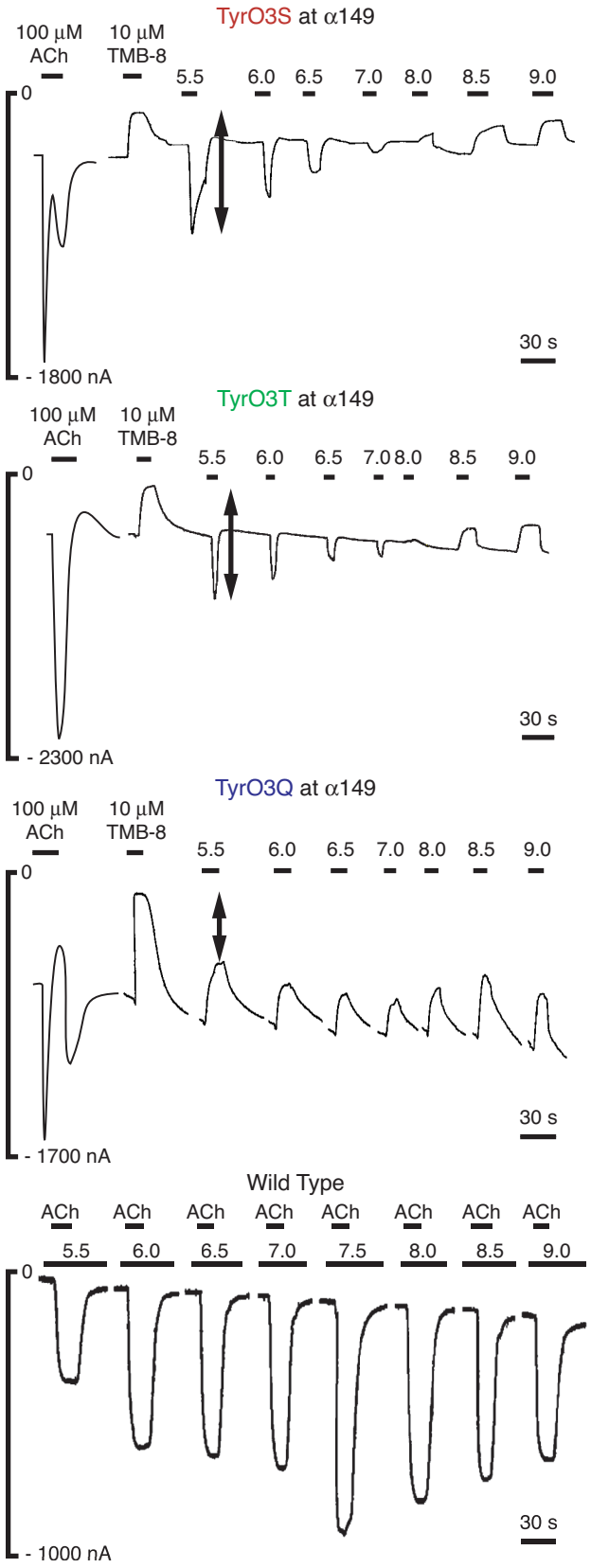

Figure A. Primary Electrophysiological Data: TyrO3S, TyrO3T, and TyrO3Q at α149: Tethered agonist responses to ACh, TMB-8, and agonist-free solutions of varying pH. Wild Type nAChR responses to 10 µM ACh at differing pHs. Upper bars indicate agonist or blocker application. Lower bars indicate application of solution of given pH. Arrows indicate blockable constitutive currents.

Primary Electrophysiological Data: Examples of electrophysiological recordings for TyrO3S, TyrO3T, and TyrO3Q suppressed at α149 are shown in Figure A. For each tethered agonist, there is a substantial standing current even at pH 7.5, which can be blocked by TMB-8. As seen in previous tethered agonist studies, added ACh causes an increase in current (followed by desensitization), indicating that the tethers are "partial agonists." For TyrO3S and TyrO3T, application of low pH agonist-free solutions potentiate constitutive activity by protonating the tether. For TyrO3Q, which is always charged, changes in constitutive activity with pH merely mirror the inherent changes in channel conductance as seen for wild type responses to 10 μM ACh at various pHs (Figure A, bottom).

TyrO3P: The primary tether incorporates and gives functional nAChRs, as seen by the response to ACh, but shows no constitutive activity, even at pH 5.5. (Figure B) It would be surprising that the tether remains unprotonated, as the pK_a of aminopropanol is 10.2, higher than the 3° amine.[5] TyrO3P may lack the steric bulk to activate the receptor.

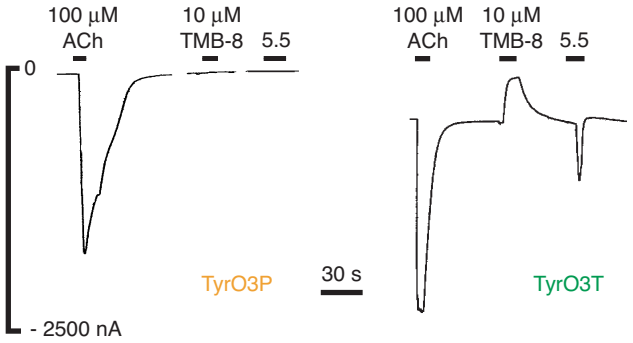

Figure B. TyrO3P shows no constitutive activity. TyrO3P and TyrO3T at α149, their current responses to ACh, TMB-8, and pH 5.5.

α149 Lys mutant: No ACh-induced or TMB-8-blockable currents were observed, and fluorescent labelling studies with tetramethylrhodamine-conjugated bungarotoxin (BuTx-TMR, an antagonist) showed no surface expression of α149 Lys mutant nAChRs in oocytes. Six days following mRNA injection, oocytes were incubated (60 min, 4°C) in ND96 solution containing BuTx-TMR (100 nM) and bovine serum albumin (5 mg/ml). After three washes with ND96 the fluorescence intensity of the animal pole was determined using an inverted epifluorescent microscope (IX-70 FLA; Olympus Corp.) equipped with a photomultiplier tube (R928P; Hamamatsu Photonics) attached to the side port. This microscope (described previously, Li *et al*., 2000) was fitted with an oil-immersion objective of 40X, NA 1.35.

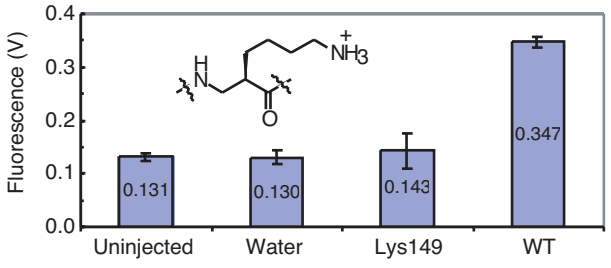

Figure C. Fluorescent labelling of α149 Lys mutant nAChRs.

Tethered agonists at sites α93 and γ55/δ57. As stated earlier, the efficacy of a tethered agonist can be evaluated in terms of the ratio of the constitutive current that can be blocked by TMB-8 to the maximum current induced by saturating concentrations of ACh. An examination of the efficacies of TyrO3S, TyrO3T, and TyrO3Q at α93 and γ55/δ57 (Figures D and E) shows that all three tethers are less potent at these sites than at α149. TyrO3T and TyrO3Q curves are similar in shape to the α149 curves. Surprisingly, although TyrO3S yields constitutive activity at all three sites, it cannot be potentiated by pH at α93 and γ55/δ57.

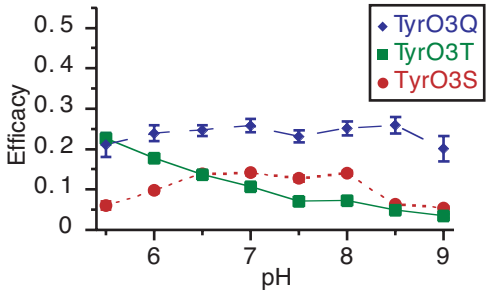

Figure D. α93: Efficacy as a function of solution pH for tethered agonists at α93. Shown on the same scale as Figure 2 in main text.

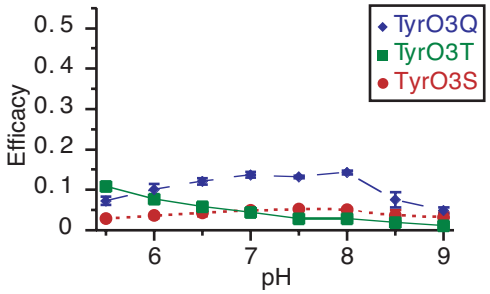

Figure E. γ55/δ57: Efficacy as a function of solution pH for tethered agonists at γ55/δ57. Shown on the same scale as Figure 2 in main text.

TyrO3*t*-Bu, an uncharged analog of TyrO3Q in which the quaternary nitrogen has been replaced by a carbon, also gave weakly constitutively active receptors at α93 and γ55/δ57 (but not at α149). TyrO3*t*-Bu showed no pH-dependent potentiation over background. This indicates that charge may be less of a factor at these positions than at α149, and may help to explain the puzzling TyrO3S behavior.

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